

IN VITRO ANTIBACTERIAL ACTIVITIES OF COCKROACH EXTRACTS AGAINST SELECTED BACTERIAL PATHOGENS

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ABSTRACT

Currently bacteria and other microorganisms continue to form resistance strains against antimicrobial agents in use hence novel sources of antibiotics are urgently needed to salvage this menace. This study investigated the *in vitro* antibacterial activity of cockroach extracts against standard reference control strains of pathogenic bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). The cockroaches used in the study were collected from kitchen store rooms, corners of buildings, household manholes, rubbish dumps and sent to the laboratory where the insects were identified as *Periplanetta americana* L., killed, and dissected. Chloroform, ethanol, and aqueous extracts of the various parts were prepared and screened against selected bacterial pathogens using Clinical Laboratory Standard Institute (CLSI) methods. Minimum inhibitory and bactericidal concentrations (MICs and MBCs) of

the most active of the extracts were also determined using CLSI methods. Ethanol extracts of the guts and exoskeletons were the only extracts of the cockroaches that showed activities with mean diameters of zones of inhibition ranging from 15-22 mm against methicillin-sensitive *Staphylococcus aureus* (MSSA), MRSA, and *Escherichia coli*. MICs and MBCs values ranging from 25-50 mg/ml and 50-100 mg/ml were found against the test bacteria. However, no activity was observed against *Pseudomonas aeruginosa* with all the cockroach extracts tested. Ethanol extracts of the guts and exoskeletons of the cockroaches investigated have shown some level of activity against the selected bacterial pathogens including MRSA. Work is on-going to isolate the bioactive component(s) of the cockroach extracts.

Key words: Bacteria, cockroach, ethanol, extract, MIC, MBC

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INTRODUCTION

Nowadays key bacterial pathogens continues to form resistant strains against currently available antimicrobial agents, there is therefore the need for novel sources such as insects pets to battle this menace. Insects can be more of a health hazards because of the behavioral

characteristics of insect pests have always raised safety concerns especially as carriers of food borne pathogens and food spoilage organisms among others (Pai *et al.*, 2005). Insects can play potential roles in the transmission of pathogenic bacteria and fungi with antibiotic resistance in households and in hospital environments (Salehzadeh *et al.*, 2007). Unsanitary environments that insect pests often live in have enabled the insects to encounter and harbor many different species of bacteria and other microorganisms. It is therefore logical that these insects may have developed ways of protecting itself against these pathogenic microorganisms. In a drive to find new sources of antimicrobial agents, scientists and researchers did focused their attention on plants and their potentials, however just like plants insects also produce complex suites of chemicals. Most insects produce these complex chemicals for various purposes including defense, mating, communication, and other processes that help the insects to survive.

Cockroaches are nocturnal omnivore's insects that live in damp places throughout the world and belong to the Order: Blattodea with most species associated with human habitats. The most commonly known as household pests includes *Periplaneta americana* L., *Blattella germanica* L., *Blattella asahinai* M., and *Blattella orientalis* L. A standard cockroach body is flattened and broadly oval with a large shield like pronotum covering the head (Anon, 2015). For example, *P. americana* has a ventrally positioned chewing mouth parts made up of a collection of appendages, a long segmented antennae and a pair of membranous wings arising from the mesothorax that are thick and leathery (Plate 1). Also the body of *P. americana* is externally covered by a hard chitinous exoskeleton secreted by underlying cells which provides surfaces for attachment of muscles and also protects the body. In the search for new antimicrobial agent(s) this study investigated the in-vitro antibacterial activity of extracts of *P. americana* against standard reference control strains of pathogenic Gram positive and negative bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS

Collection of cockroach samples

Cockroaches were collected from households, kitchen store rooms, and corners of buildings using cockroach traps at Korle-Gonno, a suburb of the Accra Metropolis, Ghana. Also some of the cockroaches were caught directly from household man-holes, rubbish dumps by using sterile surgical gloves into sterile jars with small holes on the top of the lid to provide air. The insects were then sent to the Department of Animal Biology and Conservative Science (ABCS), School of Biological Sciences, University of Ghana and were identified as *P. americana* (Class: Insecta, Family: Blattidae) by Dr. Maxwell Kelvin Billah (first author) using standard taxonomic systems. After that the cockroaches were transported to the Microbiology Laboratory (ML), School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences, University of Ghana, Korle-bu for the analysis.

Processing of cockroach samples

Dissection of cockroach samples

At the ML, SBAHS, Korle-bu the live intact matured cockroaches were killed by placing a cotton ball soaked with chloroform into the jar for 1 min. After which the insects were brought out of the jar and dissected. During the dissection the wings were initially pinned to the background of the dissection board and the legs were cut off to allow free view of the underside of the cockroaches. With the aid of sterile surgical blades the heads were cut off and slight cuts were made from the heads to the abdomens. Sterile forceps were then used to remove the exoskeletons and sterile surgical blades used to remove the fatty tissues. After that the entire guts of the cockroaches consisting of the foreguts to the hindguts were removed by the use of the sterile forceps and then placed into sterile containers. This process

was repeated until 10 g of the guts of the cockroaches were obtained. Also by the use of a dissecting microscope, sterile forceps, and surgical blades, the brains of the cockroaches were dissected out and placed into sterile containers.

Processing of the different parts

The guts and brains of the cockroaches were processed by macerating them separately in a clean dry mortar and pestle, after which the samples were transferred into clean sterile containers. Also the entire bodies (exoskeleton) of the cockroach samples were dried in a hot air oven at 45°C and then crushed into powdery form by the use of a clean dry mortar and pestle. The coarse powders were also made into fine powders by the use of a grinding stone, placed into separate sterile containers and labeled for storage prior to use for the analysis.

Extraction

A total of 10 g each of the various grounded parts of the cockroach powders were placed in separate containers and 100 ml of chloroform (absolute), ethanol (75%), and distilled water (H₂O) were added into each container separately. The contents in the containers were mixed thoroughly and capped with a tight fitting lid and then allowed to stand undisturbed overnight. After which the solutions were filtered using Whatman No. 1 filter paper (Whatman International, UK) to remove solid insect material. For the organic extracts, the chloroform and the ethanol were removed from the filtrate in *vacuo* at 37°C in a Buchi Rotavapor rotary evaporator (Rose Scientific Limited, Canada) while the aqueous extracts were freeze dried using Modulyo freeze dryer (Thermo Fisher Scientific, USA). The different solvent extracts of the guts, brains, and exoskeletons of the cockroach samples were then weighed and the dry weights and percentage yields (% w/w) calculated using the formula.

$$\frac{\text{weight of dry extract}}{\text{weight of ground powder}} \times 100\%$$

Preparation of stock solutions of the extracts

Each of the solvent cockroach extracts (1200 mg) were scrapped from the total extract obtained, and measured with the aid of a weighing balance into clean sterile containers. For the chloroform and ethanol extracts because they were organic extracts and will partially dissolve in aqueous medium few drops of dimethyl-sulphoxide (DMSO) were added to dissolve the extract after which sterile distilled H₂O were added to make up 3 ml to give a final extract concentrations of 400 mg/ml each. However, for the aqueous extracts of the millipedes, 3 ml of sterile distilled H₂O were added to the extract and then sterile filtered through 0.2 µm Millipore filters (Merck Millipore, Germany). All the prepared extracts were dispensed into vials and stored in the fridge prior to use.

Sources of the test bacterial strains

Standard control strains of bacteria were obtained from the American Type Culture Collection (ATCC), Rockville MD, USA including methicillin-sensitive *Staphylococcus aureus* (MSSA: ATCC 25923), MRSA (ATCC 43300), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 19429). The test control bacterial strains were maintained on nutrient agar slants prior to their use.

Antibacterial activity evaluation

Preparation of paper-discs

The paper-discs with the cockroach extracts used in this study were prepared using modification of the method by Cheesbrough (2006). Paper-discs (6 mm diameter) were punched out from sheets of Whatman filter papers and then placed in Petri dishes, allowing distances of 5 mm between each disc. The paper-discs were sterilized in a hot air oven at 160°C for 1 h and allowed to cool. With the aid of a micropipette with sterile tips, 20 µl of the cockroach extract with specific concentration were added onto each disc in drops

allowing it to dry intermittently before adding other drops. The paper-discs were finally dried by placing the Petri dishes containing the discs in an incubator at 37°C for 1 h. The performance of each solvent extract of the paper-discs was checked against standard control strains of bacterial pathogens using commercial antimicrobial agents.

Agar-well and paper-disc diffusion assays

For both assays, 3-5 colonies of the test bacterial strains from overnight growth cultures were transferred into 5 ml sterile physiological saline (0.85% NaCl) incubated for 2 h at 37°C, and the optical density measured using spectrophotometer (Aurora Instruments Limited, Canada) at 500 nanometers (nm). The bacterial cultures were standardized according to Clinical Laboratory Standard Institute (CLSI, 2012) methods. Sterile cotton wool swabs were used to pick the inocula for the streaking of the entire surfaces of Mueller-Hinton agar (MHA) plates rotating in 3 directions at approximately 60° for evenly distribution of inocula of the tests bacteria on the MHA plates. Then 6 mm diameter wells were created in the inoculated MHA agar plates by the use of a sterile cork borer. Using a micropipette with sterile tips, 100 µl of the different extract stock solutions of the cockroaches were dispensed into each different well in the MHA plates. However for the paper-disc diffusion assay, laboratory prepared air dried paper-discs were placed onto the surfaces of inoculated MHA plates with the aid of sterile inoculating pins. Positive and negative controls were set up for both the agar-well and paper-disc diffusion assays using known commercially produced paper antimicrobial agents obtained from Axiom Laboratories, India including gentamicin (10 µg), cotrimoxazole (25 µg), tetracycline (30 µg), and sterile distilled H₂O. All the plates with the wells and paper-discs were then allowed to stand on the bench for 15 min to allow for the diffusion of the cockroach extracts, controls, and incubated at 37°C for 18-24 h. After overnight incubations, vernier clippers were used to measure the diameters of the zones of inhibition around the

wells and paper-discs. The whole experiment was repeated for 2 more consecutive times and the mean diameters of zones of inhibition calculated for each bacteria.

Determination of minimum inhibitory and bactericidal concentrations (MICs and MBCs)

MICs and MBCs of the most active of the cockroach extracts were determined using modification of the broth dilution method by CLSI (2012). First, 100 μ l of Mueller-Hinton broth (MHB) were distributed into each of a 96-wells microtitre tray from the 1st-12th well. Using a micropipette with sterile tips, 100 μ l of the cockroach extracts were added to the 1st rows, the contents well mixed and 100 μ l of the extract and broth pipetted into the 2nd rows of the microtitre tray. These processes were repeated using serial doubling dilutions from the 2nd-11th wells leaving the 12th wells as positive growth controls. After that 100 μ l of a previously prepared inocula suspension of 10^6 colony forming units per milliliters (CFU/ml) of the test bacteria were delivered into each of the wells starting from the 1st-12th wells. Negative controls were also set up at the bottom rows of the microtitre tray consisting of only MHB alone without bacteria. The microtitre tray was covered with a sterile plastic cover and incubated for 18-24 h at 37°C. Also purity controls were set up alongside by taking loopful each of the suspensions from the positive controls wells and sub-cultured onto blood agar (BA) plates and incubated at 37°C in 3-5% CO₂ for 18-24 h. The purity plates for the positive controls were examined together with the negative controls for possible contamination prior to the reading of the MICs. MICs of the cockroach extracts were taken as the lowest concentration of the extracts showing no visible growth of the test bacteria. MBCs were also determined by sub-culturing 10 μ l from each of the wells unto nutrient agar (NA) plates and incubated at 37°C for 24-48 h. After incubation the plates were then observed for growth and the lowest concentration of the cockroach extracts that produced no visible growth on the NA

plates were taken as the MBCs. The experiments were done in duplicates for both MICs and MBCs and the mean values calculated.

RESULTS

Extracts

Percentage (%) yields of the various solvents cockroach extracts ranges from 1.2-5.20 for chloroform, 9.60-25.2 ethanol (75%), and 4.10-8.50 aqueous extracts (Table 1). The highest % yield (25.2%) was obtained for the ethanol (75%) extract of the guts of the cockroaches' while the lowest % yield (1.20%) was obtained for chloroform extract of the brains (Table 1).

Table 1. Percentage yields (%) of the various solvents cockroaches extracts

Chloroform		Ethanol		Aqueous	
Brain Body	Gut	Brain Body	Gut	Brain Body	Gut
1.20 5.20	2.60	9.60 19.8	25.2	4.10 7.20	8.50

Antibacterial evaluation

Tables 2 and 3 shows the mean diameters of the zones of inhibition of the cockroach extracts using agar-well and paper-disc diffusion assays against the different control bacterial strains

used in the study. Most of the cockroach extracts were not active against the test bacteria except the guts and exoskeletons. For example, by the agar-well diffusion assay, the mean diameter of zones of inhibition of the ethanol (75%) extract of the guts ranged from 15-22 mm against MSSA, MRSA, and *E. coli*. Also for the ethanol (75%) exoskeletons extract of the cockroaches, the mean diameter of zones of inhibition ranged from 10-14 mm against only MSSA and MRSA. It must be noted that none of the extracts of the cockroaches were active against *Ps. aeruginosa*. In the paper-disc diffusion assay, the mean diameter of zones of inhibition of the ethanol (75%) extract of the guts of the cockroaches ranged from 12-18 mm against MSSA, MRSA, and *E. coli* but was also not active against *Ps. aeruginosa* (Table 3). Mean diameter of zones of inhibition of the control antibiotics including gentamicin (10 µg), cotrimoxazole (25 µg), and tetracycline (30 µg) ranged from 12-26 mm against MSSA and MRSA (Table 4). For *E. coli* and *Ps. aeruginosa*, the mean diameter of zones of inhibition ranges from 22-27 mm. However, no activity was observed for tetracycline (30 µg) against *E. coli* and *Ps. aeruginosa* (Table 4).



Plate 1. Dorsal view of *Periplaneta americana* L. studied.

Quantitative antibacterial evaluation

The MIC and MBC values of the ethanol (75%) extract of the guts of the cockroaches are presented in Table 4. MIC and MBC values of 25 and 50 mg/ml were found against MSSA and MRSA while for *E. coli* the MIC and MBC values were 50 and 100 mg/ml respectively.

DISCUSSION

Methods used in the preparation and concentrations are very important for the determination of antibacterial activity of extracts and that the solvent system can also play a crucial role in the solubility of the materials to be extracted hence can influence the antibacterial activity of the formulated drug or extract. After the extraction of the cockroach samples, it was observed that both chloroform and aqueous extracts have the lowest % yields as compared to the ethanol (75%) extracts (Table 1). In a similar related study in Ghana using plants for the extraction processes also observed low % yields with chloroform extracts (Pesewu *et al.*, 2008).

Ethanol (75%) extracts of the guts and exoskeletons were the only parts of *P. americana* that showed activity against MSSA, MRSA, and *E. coli* but were not active against *Ps. aeruginosa* by both the agar-well and paper-disc diffusion assays in this study (Tables 2 and 3). Similar antibacterial activities were observed with the paper-disc diffusion assay but however with slightly lower mean diameters of zones of inhibition. The ethanol (75%) extract of the guts of *P. americana* showed the highest activity as compared to the other solvent extracts so was selected for quantitative antibacterial analysis. MIC and MBC values of the ethanol (75%) extract were lower against MSSA and MRSA as compared to *E. coli* (Table 5) which suggest that the guts extract of *P. americana* can be more effective at a lower concentration against MSSA and MRSA than *E. coli*. In a previous study in Nottingham, UK

the brain and nervous system of *P. americana* extracts have been found to be active against both pathogenic bacteria including MRSA (Anon, 2010). But in this study the brain extracts *P. americana* were inactive against all the test bacteria may be due to the different extraction procedures used in both studies. Antibacterial activities of extracts of *P. americana* have also been reported to be active against Gram positive bacteria but were not active against *Ps. aeruginosa* (Seraj *et al.*, 2003). This activity observed with the *P. americana* extracts may be due to the presence of induced antibacterial peptides produced by cockroaches against pathogenic bacteria found in the body (Dillon *et al.*, 2005). For it is known that cockroaches dwell in unsanitary places and therefore tends to harbor all sorts of microorganisms such as bacteria, fungi, and parasites on its body (Tetteh-Quarco *et al.*, 2013). Cockroaches use immunological responses similar to that of vertebrates to fight against pathogenic bacteria that may serve as a threat to it. These immunological responses signal complex glandular systems can secrete antimicrobial peptides against the pathogen of interest (Dillon *et al.*, 2005).

CONCLUSION

From the study, ethanol (75%) extracts of the guts and exoskeletons of *P. americana* have shown promising antibacterial properties against the test bacteria including MRSA. However work is on going in the isolation of the bioactive component(s) of the *P. americana* extracts using various analytical methods including high performance liquid chromatography (HPLC).

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Table 2. Mean diameter of zones of inhibition (mm) of the various solvents cockroaches extracts using the agar-well diffusion assay

Test bacteria	Chloroform			Ethanol			Aqueous		
	Brain	Gut	Body	Brain	Gut	Body	Brain	Gut	Body
MSSA	-	-	-	-	22 ± 0.01	14 ± 0.04	-	-	-
MRSA	-	-	-	-	20 ± 0.00	10 ± 0.02	-	-	-
<i>E. coli</i>	-	-	-	-	15 ± 0.02	-	-	-	-
<i>Ps. aeruginosa</i>	-	-	-	-	-	-	-	-	-

MSSA = methicillin-sensitive *Staphylococcus aureus*, MRSA = methicillin-resistant *Staphylococcus aureus*

E. coli = *Escherichia coli*, *Ps. aeruginosa* = *Pseudomonas aeruginosa*

- = no activity observed

Table 3. Mean diameter of zones of inhibition (mm) of the various solvents cockroaches extracts using the paper-disc diffusion assay

Test bacteria	Chloroform			Ethanol			Aqueous		
	Brain	Gut	Body	Brain	Gut	Body	Brain	Gut	Body
MSSA	-	-	-	-	18 ± 0.00	-	-	-	-
MRSA	-	-	-	-	16 ± 0.00	-	-	-	-
<i>E. coli</i>	-	-	-	-	12 ± 0.01	-	-	-	-
<i>Ps. aeruginosa</i>	-	-	-	-	-	-	-	-	-

MSSA = methicillin-sensitive *Staphylococcus aureus*; MRSA = methicillin-resistant *Staphylococcus aureus*

E. coli = *Escherichia coli*, *Ps. aeruginosa* = *Pseudomonas aeruginosa*

- = no activity observed

Table 4. Mean diameter of zones of inhibition (mm) of the control standard antimicrobial agents against the control bacteria

Test bacteria	Antibiotics		
	GEN	COT	TET
MSSA	26 ± 0.00	17 ± 0.20	22 ± 0.18
MRSA	20 ± 0.00	12 ± 0.01	14 ± 0.00
<i>E. coli</i>	25 ± 0.00	27 ± 0.00	-
<i>Ps. aeruginosa</i>	23 ± 0.01	26 ± 0.00	-

GEN = gentamicin (10 µg), COT = cotrimoxazole (25 µg), TET = tetracycline (30 µg)

MSSA = methicillin-sensitive *Staphylococcus aureus*

MRSA = methicillin-resistant *Staphylococcus aureus*

- = no activity observed

Table 5. MIC and MBC values (mg/ml) of the ethanol extract of the cockroaches and the control antibiotic

Test bacteria	Cockroach extract		Control (ciprofloxacin)	
	MIC	MBC	MIC	MBC
MSSA	25	50	0.0008	0.0008
MRSA	25	50	0.0008	0.0008
<i>Escherichia coli</i>	50	100	NA	NA

MSSA = methicillin-sensitive *Staphylococcus aureus*; MRSA = methicillin-resistant *Staphylococcus aureus*

NA = not applicable